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LOSS IN VIRULENCE OF YELLOW FEVER VIRUS SERIALLY PASSED IN HELA CELL CULTURES

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TECHNICAL MANUSCRIPT 204

LOSS IN VIRULENCE OF YELLOW FEVER VIRUS SERIALLY PASSED IN HELA CELL CULTURES

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ABSTRACT

Yellow fever virus passed in HeLa cells was characterized for adaptation to growth in cell culture, level of titer, cytopathic effect, stability to aerosolization at 2 humidities, and respiratory infectivity for monkeys. Four viral populations with different combinations of properties were obtained.

I. INTRODUCTION

Miller, et al.[1] have shown that HeLa cell cultures supported the growth of yellow fever virus (Asibi strain) and that the virus grown in such a system was capable of infecting rhesus monkeys* at low doses by the respiratory route. Hardy[2] showed that six serial passages of the Asibi strain of virus in HeLa cells resulted in a virus population that was not lethal for monkeys but lysed the cells in culture. This was in contrast to the parent mouse brain preparation, which was lethal for monkeys but did not lyse the cells in the culture. Our investigation of these phenomena revealed that serial passage of this virus in HeLa cells was attended by alterations of several more viral properties and that some of these changes took place at earlier passage intervals than had been previously reported. Moreover, some of the genetic markers of the viral populations that were encountered appeared to be closely associated with one another.

II. MATERIALS AND METHODS

A. HARVEST OF VIRUS FROM HELA CELL CULTURES

The Asibi strain of yellow fever virus was inoculated into a stock strain of HeLa cells maintained at the U.S. Army Biological Laboratories. Viral inoculum consisted of a suckling mouse brain seed preparation. In one instance a monkey plasma seed preparation was employed. Prior to inoculation of the cell cultures, the monolayers, grown in T-60 flasks, were washed once with Hanks' balanced salt solution (BSS). One ml of inoculum, diluted 1:5, was placed over the cells and allowed to adsorb for 1 hour at 37 C. The cells were washed twice with BSS and overlayed with 12 ml of growth medium consisting of yeast extract and proteose peptone 3 supplemented with 20% horse serum.[3] The medium was replaced every day and samples of the supernatant fluid were harvested and frozen at -60 C with egg yolk from 6-day-old embryonated eggs (20% v/v) until they were titrated intracerebrally in 10- to 14-gram mice. Samples that were harvested for use as aerosols consisted of the infected supernatant medium and the cells that were scraped off the glass surface mixed with 20% egg yolk.

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* In conducting the research reported here, the investigators adhered to "Principles of Laboratory Animal Care" as established by the National Society for Medical Research.

B. AEROSOL METHODS

The procedures employed to produce aerosols of the viral preparations into an atmosphere with relative humidities (RH) between 40 and 80%, the exposure of monkeys to these preparations, the assay procedures, and the method by which the initial per cent recovery of virus was determined (including replication and analysis) have been described by Miller et al.[1] For each assessment of infectivity, nine rhesus monkeys were divided into 3 groups of 3 each. Two additional monkeys were held as controls. Each group received different inhaled doses achieved by aging the virus aerosol. In the cases indicated below, monkeys that survived virus infections were challenged by the intraperitoneal (ip) route with a lethal dose consisting of 1000 mouse intracerebral (MIC) LD_{50} of the mouse brain seed.

III. RESULTS

Eleven experiments were carried out in a similar manner in order to characterize the properties of virus that had been passed once in HeLa cells. Mean values of the data are presented in Table 1 as a "typical" virus population (Ty-1). Maximal virus titers ranged from $10^{6.9}$ to $10^{7.4}$ $MICLD_{50}$/ml of virus and were obtained in 5 days; no evidence of lysis of the HeLa cells was observed. The initial virus recoveries from aerosols of three preparations were not affected by exposure to different relative humidities, as revealed by a statistical analysis at the 5% level of significance. At 40 to 50% RH, recovery values ranged from 24.6 to 40.6%; those at 70 to 80% RH were 28.8 to 41.7%. Ten $MICLD_{50}$ of these virus preparations administered to rhesus monkeys by exposure to aerosols during three representative experiments produced lethal infections. Pathologic evidence of liver involvement of infected monkeys was similar to that described for yellow fever by Tigertt, et al.[4]

In contrast to the viral populations observed during the eleven tests mentioned above, one additional population was encountered that was "atypical" (ATy-1). The activity of this population was significant because of a cytopathic effect (cpe) that was found after only one passage in HeLa cells; the data that are to be discussed later show that this type of response (i.e., cpe) was observed usually after three serial passages. Because of the rarity of this event, this virus population was studied in the same detail as the "typical" one-passage population (Ty-1) mentioned above. Properties of both Ty-1 and ATy-1 after one passage in HeLa cells are presented in Table 1.

TABLE 1. PROPERTIES OF "TYPICAL AND "ATYPICAL" YELLOW FEVER VIRUS POPULATIONS OBTAINED AFTER ONE PASSAGE IN HELA CELL MONOLAYERS

Properties	Populations	
	(Ty-1)[a]	(ATy-1)[b]
Maximal $MICLD_{50}$/ml (log_{10}) Titer in Culture	7.1	8.1
Time of Maximal Titer Postinoculation	day 5	day 5
Cytopathic Effect in Culture	negative	positive
Per Cent Initial Recovery in Aerosol		
40-50% relative humidity	43.2	9.7
70-80% relative humidity	46.7	28.2
Monkey Lethality 40-50% relative humidity	lethal at 10 $MICLD_{50}$	lethal at 10 $MICLD_{50}$

a. "Typical" viral population representative of harvests obtained in eleven of twelve experiments.
b. "Atypical" viral population representative of a harvest obtained in one of twelve experiments.

Differances shown by ATy-1 include a tenfold increase in viral yields, cell lysis in culture, and an adverse effect of the lower relative humidity. The recovery of 9.7% that was obtained at 50% RH was significantly lower ($P<5\%$) than the 28.2% that was obtained at 80% RH. Similarities between Ty-1 and ATy-1 include the 5-day postinoculation interval of maximal titer and lethality for monkeys at doses of 10 $MICLD_{50}$.

In a series of six experiments the yellow fever virus was serially passed three times in HeLa cells. Data from the six experiments revealed that populations obtained in two experiments were uniformly different from those obtained during the other four. This suggested that a greater incidence of variation was found among populations obtained after three serial passages than after one. The results of the majority (four) of experiments were tentatively considered as "typical" while those of the minority (two) were considered as "atypical." A comparison of the properties representative of the two types of third passage populations is shown in Table 2.

TABLE 2. PROPERTIES OF "TYPICAL" AND "ATYPICAL" YELLOW FEVER VIRUS POPULATION OBTAINED AFTER THREE SERIAL PASSAGES IN HELA CELL MONOLAYERS

Properties	Populations (Ty-3)[a]	(ATy-3)[b]
Maximal $MICLD_{50}$/ml (log_{10}) Titer in Culture	8.8	6.9
Time of Maximal Titer Postinoculation	day 3	day 3
Cytopathic Effect in Culture	positive	negative
Per Cent Initial Recovery in Aerosol		
50% relative humidity	9.8	19.7
80% relative humidity	31.5	12.3
Monkey Lethality 80% relative humidity	nonlethal at >52.5 $MICLD_{50}$	nonlethal at 800 $MICLD_{50}$

a. "Typical" viral population representative of harvests obtained in four of six experiments.
b. "Atypical" viral population representative of harvests obtained in two of six experiments.

The "typical" population (Ty-3) differed from the "atypical" population (ATy-3) by showing higher titers, cell lysis, and an adverse effect of 50% RH. The two populations were similar in demonstrating maximal yields at day 3 and by not being lethal for monkeys.

Not included in Table 2 are data suggesting that infection occurred in the absence of lethality. Monkeys that had survived the exposure to aerosols of virus resisted a challenge with a lethal dose of virus administered by the intraperitoneal route, thereby indicating that the primary respiratory infection was immunogenic.

IV. DISCUSSION

The conversion of the "typical" first-passage population, Ty-1, into the "typical" third-passage population, Ty-3, in these experiments was undoubtedly similar to the phenomenon that Hardy reported after six passages in HeLa cells.[8] The appearance of the "atypical" first-passage and the "atypical" third-passage populations, ATy-1 and ATy-3, respectively, suggest that intermediate populations can occur during that conversion. Such populations might account for the unexplained difficulty that Hardy experienced in stabilizing the virus until six passages had been performed.[8] Factors responsible for the selection of ATy-1 or ATy-3 rather than Ty-1 or Ty-3 in culture remain undefined, but unpublished data suggest that the conversion of Ty-1 to Ty-3 is facilitated by using high multiplicities of infection during serial passages of the virus. The intermediate populations appeared unstable in HeLa cells but displayed some order in their appearance. ATy-1, apparently the rarest of all the populations, shared certain of its properties with Ty-1 and Ty-3. ATy-3, which was encountered on two of six occasions, shared certain of its properties with Ty-1 and Ty-3. Surprisingly, perhaps, ATy-1 and ATy-3 shared no properties with each other.

Of considerable interest is the fact that many of the properties that were established for the various viral harvests appear to be very closely related with one another. An attempt to represent this is shown in Table 3. As examples: (i) Virus grown after one passage in HeLa cells (approximately 10^7 $MICLD_{50}$ in five days) was virulent for monkeys. Conversely, virus grown in HeLa cells after three serial passages (10^8 or greater $MICLD_{50}$ in three days) was attenuated for monkeys. (ii) Virus that had shown a cpe was adversely affected by aerosolization at 50% RH, but virus that did not induce a cpe was unaffected. (iii) Viral harvests that failed to show maximal titers in excess of 10^7 $MICLD_{50}$, despite some degree of adaptation in HeLa cells (maximal titer at day 3), did not induce a cpe; harvests that contained titers of 10^8 $MICLD_{50}$ or greater induced a cpe in culture.

One aspect of safety in the laboratory might be mentioned as a result of these studies. As indicated by previous studies[1,5-7] infections contracted in the laboratory, the inhalation of infectious virus, unaltered by laboratory passage, may constitute a danger to laboratory workers. Our studies with yellow fever virus suggest that after serial passages in vitro, the danger of airborne infections may be markedly reduced. This could be attributed not only to the selection in vitro of particles

TABLE 3. A SUMMARY OF CHANGES IN THE PROPERTIES OF YELLOW FEVER VIRUS (ASIBI STRAIN) IN HELA CELL CULTURES

HeLa Cell Preparation	Maximal $MICLD_{50}$/ml (log_{10}) Titer in HeLa Cells	Day of Maximal Titer	Cytopathic Effect[a]	Effect at 50% RH[b]	Attenuation of Virus for Monkeys[c]
One passage	6.9 to 7.4	5	negative	resistant	negative
	8 or >	5	positive	sensitive	negative
Three passages	6.8 to 7.0	3	negative	resistant	positive
	8 or >	3	positive	sensitive	positive

a. Cell rounding, increase in density, and detachment from glass.
b. Effect of aerosolization on virus at 50% relative humidity.
c. By exposure to infected aerosols.

possessing a lowered virulence, but also to the eventual selection of mutants that are unstable when airborne unless they are in an environment possessing a very high relative humidity. Whether this information can apply to other arboviruses must await the results of further tests. Evidence for a general decline in virulence in vivo of at least one other arbovirus by the respiratory as well as by other routes as a result of laboratory passage in vitro, however, has been reported.[8-9]

V. SUMMARY

Yellow fever virus that was passed one and three times in HeLa cells was characterized with respect to the following: (i) adaptation to growth in cell culture as indicated by the time required to obtain maximal titer, (ii) level of titer, (iii) production of a cytopathic effect (cpe), (iv) stability of virus when aerosolized at either 50 or 80% relative humidity (RH), and (v) the loss of virulence of viral preparations for rhesus monkeys infected by the respiratory route. Four viral populations that possessed different combinations of these properties were obtained. In eleven of twelve experiments, one passage in cell culture yielded a virus population (Ty-1) that titered to 10^7 $MICLD_{50}$/ml on day 5, that failed to cause cell lysis (cpe), that was equally stable at 50 and 80% RH, and that was lethal for monkeys. In contrast, in four of six experiments, three passages in cell culture yielded a virus population (Ty-3) that titered 10^8 $MICLD_{50}$ or greater on day 3, that induced a cpe, that had a greater sensitivity to 50 than to 80% RH, and that was nonlethal for monkeys. In the remaining experiments, one "atypical" viral population (ATy-1) was obtained from culture after one passage. In two other experiments, "atypical" viral populations (ATy-3) that were similar to each other but different from ATy-1 were obtained after three passages. The characteristics demonstrated by ATy-1 and ATy-3 suggested that these were intermediate to Ty-1 and Ty-3. The manner in which population characteristics were altered during the conversion of Ty-1 to Ty-3 in vitro suggested that some of the characteristics were closely associated with one another.

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